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Non Invasive Prenatal Diagnosis of Down Syndrome

Dimitra Kappou[1], Eleftheria Papadopoulou[2] and Stavros Sifakis[1]
[1]*Department of Obstetrics & Gynecology, University Hospital of Heraklion, Crete*
[2]*Department of Pediatrics, University Hospital of Heraklion, Crete*
Greece

1. Introduction

Down syndrome (trisomy 21), which has an incidence of 1 in 800 live births, is considered to be the most frequent etiology of mental retardation and it is the predominant reason for women seeking prenatal diagnosis [Driscoll & Gross, 2009]. Trisomy 21 is used as a benchmark because it is the most common aneuploidy compatible with life and is associated with mental retardation and serious congenital anomalies. Currently used screening tests for aneuploidy are based on the assessment of fetal sonographic markers and/or the evaluation of biochemical markers in the maternal circulation during the first and second trimester. Screening test based on the combination of nuchal translucency assessment and biochemical markers at 11^{+0}-13^{+6} weeks of gestation may detect 90-94% of pregnancies affected by Down syndrome at a false positive rate of 5% [Kagan et al., 2008]. The current gold standard for diagnosis of trisomy 21 is provided by invasive sampling of fetal genetic material through chorionic villus sampling (CVS) or amniocentesis followed by conventional cytogenetic or DNA analysis; however, both procedures are associated with an increased risk of fetal loss of about 1% and therefore they are recommended for pregnancies considered to be at high risk of fetal trisomy 21 [Alfirevic et al., 2003].

Since 1997, when cell free fetal DNA in maternal circulation was discovered, the research interest has focused on the development of reliable techniques for non-invasive prenatal diagnosis (NIPD) that would allow the direct analysis of fetal genetic material based on the discovery of cell-free fetal (cff) DNA and RNA in the maternal circulation. Current investigation fields of NIPD include fetal Rhesus D genotype determination in RhD negative women, fetal sex determination for sex-linked disorders and the role of cffDNA in pregnancy disorders such as preeclampsia but the holy grail of NIPD remains the detection of fetal aneuploidies [Honda et al., 2002; Bianchi et al., 2005]. The direct analysis of circulating fetal DNA for the NIPD of chromosomal aneuploidies is mainly complicated by the presence of the coexisting background maternal DNA. NIPD will hopefully overcome the limitations of the currently used methods for diagnosing Down syndrome antenatally and make prenatal testing safer for pregnant women and their fetuses. However, irrespective of which strategy is selected for isolating or distinguishing fetal genetic material in maternal plasma, the small quantity of cffDNA and cff mRNA poses severe technical challenges; all these issues should be addressed before the clinical application of these methods as screening test with high sensitivity, specificity and reproducibility.

In this chapter, we focus on recent advances in the NIPD of Down syndrome via the use of fetal cells or cell-free nucleic acids, and provide an overview of the future perspectives in terms of improvement of enrichment technologies and assaying methods and possibilities for clinical applications as well.

2. Intact fetal cells and cell-free fetal DNA in the maternal circulation

It has been established for over a century that fetal cells circulate in the maternal blood throughout gestation although the rarity of these cells limits eventually the practicability of a diagnostic process based on their enumeration in maternal plasma. A considerable challenge for the researchers in this field is to enrich or isolate these rare fetal cells, either for cytogenetic analysis by fluorescence in situ hybridization (FISH) or for analysis of fetal cell DNA by other molecular techniques [Bianchi and Hanson, 2006; Mavrou et al., 2007]. Several studies have reported that the number of fetal cells in maternal blood is markedly increased up to six times in women bearing aneuploid fetuses [Falcidia et al., 2004]. A large-scale study for this cell-based approach, conducted by the National Institute of Child Health and Human Development in the USA, demonstrated that detection of trisomy in these fetal nucleated erythrocyte cells is difficult possibly due to the fact that the chromosomes in these cells disintegrate some time before the nucleus is eliminated from the cell, making FISH analysis of samples from maternal circulation unreliable [Bianchi et al., 2002; Babochkina et al., 2005]. The use of fetal cells other than fetal nucleated red blood cells also found in maternal circulation has been studied, however these cells are able to persist for years, or even decades, following previous pregnancies and this persistence limits their potential value for NIPD [Guetta et al., 2003]. Possible explanations include a simple presence of these fetal cells or the fact that the maternal hematopoietic system becomes engrafted with fetal stem cells during pregnancy [Puszyk et al., 2008].

The first experimental demonstration of cffDNA in the maternal plasma and serum of women carrying male fetuses by Lo et al., (1997), opened up new possibilities in NIPD. Lo et al. were inspired by previous reports that documented the presence of tumour-derived DNA in the plasma of women suffering from a variety of cancer types. Potential sources of cffDNA include the fetal nucleated red blood cells which undergo apoptosis in the maternal circulation but the most likely source of origin is the placenta [Alberry et al., 2007]. Paternally derived DNA sequences in cffDNA can be reliably identified in maternal plasma, from as early as 5 weeks after conception and there is a positive correlation with gestational age. In particular, the median values of the quantity of cffDNA are 15.9, 21.5 and 52.0 genome equivalents/ml of blood in the first, second and third trimester respectively; the accumulation of cffDNA as pregnancy progresses lends further support to the placental origin of cffDNA [Lo et al., 1998; Sekizawa et al., 2001; Birch et al., 2005]. In particular, cytotrophoblasts (CTBs) are a likely candidate as a source of cffDNA; however, the increased rate of hyperploidy in these cells and the yet unknown relationship between the ploidy status of these cells and the ploidy of cffDNA in maternal circulation could make a diagnostic test problematical [Weier et al., 2005]. Moreover, there is a link between hypoxia and an increasing release of cffDNA that led to the suggestion that it may be a useful biomarker to assess well-being of the placenta during pregnancy [Tjoa et al., 2006]. It is known that cffDNA represents a mean of 3-6% of the DNA that is present in maternal plasma while the bulk of the DNA is derived from the mother herself and a rapid clearance occurs post partum with a half-life in the order of 16 minutes despite the narrow conflicting

results [Invernizzi et al., 2002; Rijnders et al., 2004]. First applications of cffDNA included prenatal determination of fetal sexing based on paternally derived DNA sequences such as SRY, determination of Rhesus-D status of the fetus and detection of paternally inherited genetic abnormalities [Lo, 2006; Van der Schoot et al., 2006].

3. Detection of trisomy 21 from nucleic acids in the maternal plasma

There are two approaches for the detection of trisomy 21 based on the use of cffDNA in maternal circulation. The first is defined as the relative chromosome dosage (RCD) method and compares the quantity of a chromosome 21-derived DNA sequence in cffDNA with the amount of a reference DNA sequence in cffDNA derived from a chromosome other than chromosome 21 [Lo et al., 2007a]. In a normal pregnancy the RCD of chromosome 21 is 2:2, whereas in trisomy 21 the RCD is expected to be 3:2. The second method is defined as the allele ratio (AR) method and involves the allelic ratio of single nucleotide polymorphisms (SNPs) present in a fetal-specific nucleic acid marker [Tong et al., 2006]. In a normal pregnancy where the fetus is heterozygous for a particular gene sequence, the AR in cffDNA is expected to be 1:1 whereas in a case of trisomy 21 the AR of chromosome 21 would be 2:1. The main disadvantage of this approach is that it is applicable only to heterozygous fetuses for the analyzed SNP.

3.1 Fetal DNA enrichment methods

The detection of fetal chromosomal aneuploidies with the aid of cffDNA presents considerable technical challenge: first, to select a subset of nucleic acid in maternal plasma that is completely fetal specific and second, to determine the chromosomal dosage in this subset. The major technical challenge that makes NIPD a demanding task is that cffDNA makes up a low proportion in maternal plasma in a high background of maternal DNA. Currently, several assay procedures are developed in order to enrich and enhance the fractional concentration of fetal DNA or just to distinguish the cffDNA in maternal blood samples. One point of differentiation between cffDNA and cell-free maternal DNA (cfmDNA) is that the first has a shorter size distribution (the majority being 145 bp in length or shorter whereas cfmDNA is significantly longer) [Li et al., 2004]. Based on this observation, researchers try to apply methods of size fractionation with the aid of various kits and columns that rely on the inability of large molecular weight DNA to pass through or by retention of low molecular weight DNA in a gel or column [Legler et al., 2007]. Main disadvantages of this approach are: a) the currently used electrophoretic method is labor-intensive and probably prone to contaminations and b) it is unknown if the provided DNA enrichment is enough satisfactory for the prenatal diagnosis of chromosomal aneuploidies [Lo, 2008]. In 2004, Dhallan's group proposed a specific blood processing protocol in which the addition of formaldehyde in maternal blood samples before centrifugation dramatically increased the percentage of fetal DNA recovered with the concurrent suppression of the maternal DNA background [Dhallan et al., 2004]. There are two speculations about the role of formaldehyde in increased yield of fetal DNA: a) prevention of maternal cell lysis and subsequent reduction of the amount of cfmDNA, and b) prevention of the degradation of cffDNA via its nuclease inhibitory effect [Dhallan et al., 2004]. The same research group supported that the application of this technique resulted in a significant increase in the proportion of cffDNA present from a maximum of about 6% to mean values of 20.2-25% in samples collected during various stages of gestation [Dhallan et al., 2004]. In an attempt to

reproduce these results, other investigators confirmed the previous results and reported a similar or a less pronounced increase of cffDNA (1-3%) whereas other studies yielded inconsistent results [Costa et al., 2004; Chinnapapagari et al., 2005]. A possible reason for this discrepancy is that the sample processing time differs between the studies and it is known that the amount of time spent in the tube affects the concentration of total cell-free DNA [Zhang et al., 2008]. Zhang et al. (2008) proposed that the formaldehyde addition will offer a beneficial effect if there is a delay > 6 hours in sample processing as they demonstrated no maternal blood lysis or released extra maternal free DNA into plasma within the first six hours. Future studies should be conducted to clarify the contribution of elapsed time between blood-taking and processing on the recovery of cell-free DNA from maternal plasma and determine other confounding factors in the effect of formaldehyde. The quantification of cffDNA in maternal circulation from women carrying Down syndrome fetuses could also serve as a prognostic marker for trisomy 21 as quantitative aberrations in biochemical markers of placental origin that contribute to the aneuploidies screening tests. Previous studies present conflicting results as both a two-fold increase and no significant difference in maternal concentration of cffDNA have been reported [Lee et al., 2002; Spencer et al., 2003]. Possible explanations of the observed discrepancy between the reported results include the small number of samples examined, the variable degree of placental apoptosis, the broad ranges of cffDNA concentration at each stage of pregnancy and other sampling or methodological variables that might affect the level of circulating cffDNA. In a recent study, DNA from pre-CVS maternal samples was extracted from 72 trisomy 21 and 264 control pregnancies and authors concluded that there is no difference in first trimester cffDNA levels and the quantification of cffDNA (studied only in pregnancies with male fetuses) has no prognostic value at least in the early stages of pregnancy [Gerovassili et al., 2007]. However, quantification of cffDNA in maternal plasma might be a valuable second-trimester serum marker of Down syndrome pregnancy. Farina et al., found that the maternal serum fetal DNA concentrations were elevated in 15 Down syndrome cases during the second trimester and that fetal DNA could give a 21% detection rate at a 5% false positive rate; in addition, fetal DNA increased the estimated detection rate of quadruple test from 81% to 86% at a 5% false positive rate [Farina et al., 2003]. Main limitation of this approach is that its screening performance has been evaluated only in pregnancies with male fetuses with the aid of unique DNA sequences on the Y chromosome and when a reliable gender-independent fetal DNA marker will be assayed, its clinical utility should be reassessed.

3.2 The role of epigenetic markers in rapid detection of Down syndrome

The term epigenetics refers to the molecular processes that affect gene expression with the concurrent avoidance of any change in DNA sequence or content. The most studied epigenetic process is the DNA methylation, which involves the addition of a methyl group to the cytosine residues of a DNA sequence and when it occurs in the promoters of genes has an inhibitory effect on the gene expression. Epigenetic markers for cffDNA have been discovered for other aneuploidies; these are *SERPINB5* (serpin peptidase inhibitor, clade B, member 5; also known as maspin) on chromosome 18 and *RASSF1A (*Ras association [RaIGDS/AF-6) domain family 1] on chromosome 3 [Lun et al., 2007]. In addition, the allellic ratio for placental-derived hypomethylated *SERPINB5* molecules in maternal plasma was further shown to be valuable in the non-invasive detection of trisomy 18 [Tong et al., 2006]. Nowadays, there is intense interest to identify differentially methylated DNA patterns on chromosome 21 between the placenta and maternal blood cells in order to

develop a similar method for the NIPD of Down syndrome. Such epigenetic markers could be useful either via the analysis of the epigenetic allelic ratios or directly compared with a placenta-derived DNA methylation marker on a reference chromosome [Tong et al., 2006].
A potential issue for any epigenetic approach to NIPD is the interindividual epigenetic variation as it has been documented in monozygotic twins; moreover, this process is regulated in a dynamic manner as epigenetic differences seem to increase over time in a process described as ''epigenetic drift''. However, epigenetic biomarkers sequences whose methylation has a functional significance may be subject to less individual variation than others with no functional constraint. The discovery of a number of DNA sequences that are differentially methylated between maternal and fetal DNA could provide novel markers for cffDNA via the quantification of fetal-specific DNA sequences derived from chromosome 21. One previous study described the methylation status for chromosome 21 in placenta and blood samples after the selection of sequences in promoter and non-promoter regions but it relied on an assay that used a methylation-sensitive restriction enzyme, HpaII that enables the analysis of a small proportion of all the CpG sites in the human genome [Old et al., 2007]. Differentially methylated sequences located at 21q22.3 (AIRE, SIM2 and ERG genes), 1q32.1 (CD48 gene and FAIM3 gene), 2p14 (ARHGAP25 gene) and 12q24 (SELPLG gene) were identified. Moreover, it was demonstrated that the methylation status for the sequences tested was not altered between early and term pregnancy [Old et al., 2007]. Recently, Chim et al. have performed a systematic search of 114 studied genomic regions (CpG islands) on chromosome 21 in a search for loci that were differentially methylated in placental tissue and blood cells and identified 22 (19%) that showed epigenetic differences between the maternal and fetal tissues [Chim et al, 2008]. The next step was to propose two new fetal-DNA epigenetic markers, *U-PDE9A* and *U-CGI137* found in the maternal circulation only during pregnancy and rapidly cleared upon delivery of the fetus [Chim et al, 2008]. This research group used a high resolution approach via bisulphite sequencing that increased the number of applicable CpG sites by 5-fold compared with the above-mentioned HpaII-based approach. These promising results suggest that fetal-specific epigenetic markers on chromosome 21 may provide a rich source of markers for NIPD. A novel method of trisomy 21 detection measures the ratio of a fetal-specific epigenetic marker on chromosome 21 (the putative promoter of the holocarboxylase synthetase (HLCS) gene) that is hypermethylated in the placenta and a genetic marker (ZFY, zinc finger protein, Y-linked) to determine the chromosome-dosage comparison in 5 maternal plasma samples from women carrying a fetus with Down syndrome [Tong et al., 2010]. Instead of ZFY, any other Y-chromosomal markers or any fetal-specific genetic targets that will be applied in female fetuses could also be used. Also, the placenta-specific epigenetic signature could be combined with the RNA transcripts of placental origin. This epigenetic-genetic chromosome dosage approach appears to be more precise compared to an approach based purely on epigenetic markers that will be extensively affected by the variability in the level of DNA methylation of individual molecules. Another group of investigators presented an alternative approach using methylation-dependent immunoprecipitation (MeDiP) that captures methylated sites combined with real-time quantitive PCR and identified 14 trisomy 21 cases and 26 euploid controls from pregnancies of 11-14 weeks old [Papageorgiou et al., 2011]. The accurate diagnosis of fetuses with Down syndrome was based on the ratio of a subset of fetal-specific methylated regions located on chromosome 21 compared with normal cases and regarding the clinical performance of the method both the sensitivity and

specificity were 100% [Papageorgiou et al., 2011]. The main methods performed for the study of DNA methylation are methylation-dependent immunoprecipitation (MeDiP), bisulphite conversion of DNA, and methylation sensitive restriction endonuclease assay to digest away the maternal sequneces. Main limitations of the most commonly used methods for DNA methylation analysis are that the use of bisulphite-based reagents results in DNA degradation (up to 96%) and thus in reduction of target DNA available for subsequent analysis and the methylation sensitive restriction endonuclease assay is limited to the differentially methylated regions that contain a restriction site [Grunau et al., 2001].

4. Chromosome 21-encoded mRNA of placental origin in maternal circulation

In 2000, Poon et al. showed that mRNA transcribed from the Y chromosome could be detected in the plasma of women carrying male fetuses [Poon et al., 2000]. Since then, a series of reports confirmed that cell-free fetal m RNA (cffRNA) circulates in the maternal plasma in a relatively protected form and is predominately placental in origin; therefore, it could be valuable in NIPD for Down syndrome [Tsui et al., 2002]. The underlying mechanisms by which mRNA appears in the maternal plasma remain unknown, although programmed cell death (apoptosis) seems to be involved. The transfer of cffRNA is unidirectional from the placenta to the maternal circulation and microarray-based studies of the placenta are conducted to investigate the global m RNA expression profiles in placenta, a tissue type that is only present in the fetus [Maron et al., 2007]. The m RNA transcripts of two genes expressed in the placenta, human placental lactogen (h PL) and human chorionic gonadotrophin (β HCG) have already been detected and quantified throughout gestation in maternal circulation [Chiu et al., 2006].

The potential utilization of cffRNA in detecting fetal trisomy is based on the assumption that the allelic ratio in mRNA matches the chromosomal AR; therefore, the research interest is focused on the discovery of single nucleotide polymorphisms (SNPs) as biomarkers that will exhibit the 2:1 ratio of alleles in trisomy 21 to ascertain the aneuploidy status [RNA-SNP allelic ratio approach]. Candidate m RNA markers should be encoded from genes located on chromosome 21 and be detectable in maternal plasma during early pregnancy. The first valuable cffRNA marker shown to be highly accurate in assessing trisomy 21 is PLAC4 (placenta-specific 4) m RNA transcribed from the PLAC4 gene on chromosome 21 and originating exclusively from fetal cells in the placenta and cleared following delivery of the fetus [Lo et al., 2007 b]. If the fetus is euploid, that is containing two copies of chromosome 21 and thus two copies of the PLAC4 gene, the ratio of the two candidate SNP alleles would be 1:1. Similarly, the ratio of placental mRNA in maternal plasma that is transcribed from each of these two alleles would also be 1:1. However, if the fetus has trisomy 21, then the RNA-SNP allelic ration would become 1:2 or 2:1 [Lo, 2009]. Compared with the epigenetic approach, the evaluation of RNA-SNP allelic ratio has two advantages; first, the transcription of a gene in the placenta will produce multiple copies of m RNA and second, application of reverse transcriptase PCR to detect m RNA markers is less complicated technique than bisulfite conversion methods used for the identification of epigenetic markers. The main drawback of this RNA-SNP allelic ratio approach is that it relies upon the fetus inheriting two different SNP alleles in a region which is transcribed into m RNA and therefore only fetuses heterozygous for the analysed SNP can be successfully diagnosed. Another candidate gene for this purpose is LOC90625 within the Down syndrome critical region that is over expressed in trisomy 21 placentas even from the first

trimester [Oudejans et al., 2003]. RNA from this chromosome was found to be present in 60-100% of maternal samples depending on the volume of plasma sample analysed leading to the conclusion that the detection of encoded m RNA could be used in NIPD.
This approach seems to be quite promising as in a recent study Lo et al. recruited a sample of 119 pregnancies and trough the use of a mass spectrometry-based method for measuring the RNA-SNP allelic ratio precisely, demonstrated that this strategy could achieve a high diagnostic sensitivity and specificity for trisomy 21 (90% and 96.5% respectively) [Lo et al., 2007b]. The application of novel molecular techniques as digital PCR in which individual target molecules are amplified will possibly improve the protocols for plasma RNA processing and extraction and further increase the diagnostic yield. This method could also be useful in the detection of other fetal-derived m RNA species in maternal plasma. Hopefully, the reproducibility of these success rates maybe with the addition of other markers of similar value to that of PLAC4 in large-scale clinical trials will open up new avenues in NIPD.

5. Novel techniques for the prenatal detection of Down syndrome

The urgent need for the widespread application of NIPD in the detection of trisomy 21 has created strong interest in rapid and accurate single-molecule counting methods [digital PCR, multiplexed maternal plasma sequencing] which could be used in routine clinical diagnosis in the form of automated platforms. These methods will be gender-and polymorphism-independent and will detect trisomy 21 cases based on the presence of an elevated amount of chromosome 21 sequences in maternal blood. The main disadvantage of these approaches is that they require the counting of an extremely large number of molecules for markers that are not fetal-specific (random sequences from chromosome 21) and their use demands expensive equipment and reagents and complex bioinformatics methods. We present an overview of the currently proposed techniques that have been associated with encouraging results in the detection of fetuses with Down syndrome and will hopefully be moved into the practical application.

5.1 Digital polymerase chain reaction (PCR) technology

The above-mentioned approaches (the fetal enrichment techniques, the epigenetic markers, the RNA-SNP allelic ratio method) try to resolve the issue of the low fractional concentration of fetal DNA in maternal circulation and the technical challenges that it poses in the direct detection of chromosomal aneuploidies with conventional methods, for example by real-time PCR [Lo et al., 1998]. Recent reports have indicated that digital PCR, a method that was initially applied in the determination of the allelic frequencies of oncogenic alterations in samples from patients with cancer, could be a valuable new tool in NIPD of trisomy 21 [Zimmermann et al., 2008]. In 2007, Lo et al., used digital PCR to discriminate trisomy 21 placental DNA samples from euploid ones after having applied this method in the measurement of the RNA-SNP allelic ratio for non-invasive detection of fetal aneuploidy in microwell plates [Lo et al., 2007]. In particular, they were able to distinguish four aneuploid fetuses from nine normal ones based on the PLAC4 m RNA SNP approach. In their second trial, the same research group applied a dosage approach and compared the dosage of a locus on chromosome 21 to a locus on the reference chromosome 1 and tried to detect fetal aneuploidy in artificial mixtures of euploid and aneuploidy DNA with as low as 25% trisomic material (a concentration that could be obtained in clinical samples using

enrichment strategies for cffDNA sequences). The innovation of digital PCR is that multiple PCRs are performed in parallel and each PCR will contain either a single or no target molecule. Subsequently, the counting of the number of the positive reactions at the end of amplification will lead to the estimation of the number of input target molecules. The same research group has also defined the number of molecules needed for trisomy detection in different fetal DNA concentrations. Digital PCR does not depend on allelic distribution or gender and is able to detect signals in the presence of mosaics or contaminating maternal DNA; the widespread application of this method is still limited by that the fact that a large number of digital PCRs are needed for each analysis [Fan and Quake, 2007]. Fan and Quake used for their assay material obtained from a cell line with trisomy 21 and genetic material from cells with a normal genomic complement on a microfluidic chip [Fan and Quake, 2007]. The next step was to compare the dosage of an amyloid gene sequence on chromosome 21 to that of the GAPDH [glyceraldehydes 3-phosphate dehydrogenase) on chromosome 21 which was used as reference sequence. Their preliminary results suggest that digital PCR should be indicated for the discrimination between aneuploid and normal samples. It is noteworthy that the discrimination was possible even when the aneuploidy material represented a low proportion (10%) of the total material being examined, indicating the clear advantage of this method over conventional techniques such as real-time PCR or fluorescent quantitive PCR (QF-PCR). A significant barrier for using digital PCR is the small fraction of cffDNA in maternal plasma but an approach like size-fractionation that enriches cffDNA could overcome it. So far the reported encouraging results come from few preliminary studies and the question whether this method could be introduced as a screening tool has not yet been answered. In the near future, it seems likely that the efficacy of novel applications as microfluidic digital PCR and emulsion PCR that allow the simultaneous performance of few thousands of reactions in a single PCR step will be assessed [Zimmermann et al., 2008].

5.2 Shotgun sequencing DNA

Shotgun sequencing DNA technology is based on the massively parallel sequencing of DNA that produces tens of millions of short sequence tags in a single run followed by mapping to the chromosome of origin and measurement of the over- and underrepresentation of chromosomes from an aneuploidy fetus [Mardis 2008]. Fan et al., used this method and successfully identified all nine cases of trisomy 21 in their study population at gestational ages as early as the 14th weeks [Fan et al., 2008]. Further studies are required to specify technical features as the sample-volume limitations and the variations in the counts of sequenced fragments from sample to sample; in addition, this technology will contribute to current knowledge about cell-free nucleic acids revealing unknown features about plasma mRNA distributions and epigenetic features of plasma DNA.

5.3 Multiplexed maternal plasma DNA sequencing

Multiplexed maternal plasma sequencing can overcome the difficulty that poses the small proportion of fetal DNA in maternal circulation as it can identify and quantify millions of DNA fragments in biological samples in a span of days [Schuster 2008]. The feasibility and the diagnostic performance of this alternative approach has already been explored in three cohort studies that recruited few Down syndrome cases with promising results [Chiu et al., 2008; Fan et al., 2008; Chiu et al., 2010]. A recent large-scale validity study used multiplexed maternal plasma DNA sequencing analysis in 753 pregnant women at high risk for fetal

trisomy 21 according to the results of conventional screening who underwent invasive procedures for full karyotyping [Chiu et al., 2011]. Two different protocols (2-plex protocol and 8-plex protocol) were used with different levels of sample throughput followed by the measurement of the proportion of DNA molecules that originated from chromosome 21. The 2-plex protocol achieved 100% sensitivity and 97.9% specificity to rule out trisomy 21 with a positive predictive value of 96.6% and negative predictive value of 100% while the 8-plex protocol with which less plasma DNA molecules were analyzed, exhibited a relatively moderate diagnostic performance. The researchers also concluded that if the referrals for amniocentesis or CVS were based on the sequencing tests results, invasive diagnostic procedures could be avoided in about 98% of the cases in a high-risk population [Chiu et al., 2011]. Further studies will confirm the suitability of this method as first trimester screening test in the general population and its cost-effectiveness as it is currently expensive and not easily accessible to diagnostic laboratories.

6. Prenatal detection of Down syndrome through detection of trophoblasts in cervical smears

Fetal cells are also present in the uterine cavity from 5 to 15 weeks of pregnancy and are most probably exfoliated extravillous trophoblasts (shed from the placenta) [Holzgreve and Hahn, 2000]. Trophoblast cells can be retrieved from the cervical canal using aspiration, cryobrush or cotton wool swabs, endocervical lavage, and intrauterine lavage. Initial approaches using endocervical samples obtained by mucus aspiration or by cryobrush resulted in higher success rates of fetal sex prediction [Griffith-Jones et al, 1992; Falcinelli et al, 1998]. However, direct PCR amplifications from unpurified transcervical cells are likely to result in maternal cell contamination. A more recent study using PCR and FISH analyses on transcervical cells resulted in poor detection of fetal cells [Cioni et al, 2003]. To distinguish trophoblast cells from the predominant maternal cell population in transcervical cell samples, antibodies directed against placental antigens were employed [Koumantaki et al, 2001; Bulmer et al, 2003]. These analyses resulted in an overall detection rate of trophoblasts of 25 to 93%.

Another suggestion is that fetal cell search can be improved through better and faster recognition of fetal cells with the aid of automated scanning (automated microscope systems). Theoretically, the automated microscope could work faster and continuously and thus process more cells or more samples than the fatigued human. Analysis of interphase nuclei by FISH, can be used to detect numerical chromosome aberrations (Evans et al., 1992; Ward et al., 1993). The attraction of FISH as a relatively simple approach is based on experience with peripheral blood, amniocentesis samples, and transcervical samples, which have large numbers of cells to examine so that occasional poor signals are only a nuisance. With the few fetal cells available in fetal cell work, FISH quickly shows limitations. It seems necessary to further develop automated microscope systems, which would robotically identify and analyse putative target fetal cells. A recent study tested the hypothesis that fetal cells retrieved from the distal endocervical canal during the first trimester (as early as 5 weeks) may be a source of fetal genetic material for NIPD of trisomy 21 [Sifakis et al., 2011]. The hybridization of fetal cells with chromosome 21 specific probes followed by analysis with an automated fluorescence microscope led to the successful detection in 5 out of 5 trisomy 21 pregnancies [Sifakis et al., 2011]. Examples of the trisomy 21 cells detected, one from a male and the other from a female trisomy 21 pregnancy are shown in the Figure 1.

Additional studies with larger sample size are required to verify the potential of the utilization of fetal cells obtained via cervical samples for NIPD.

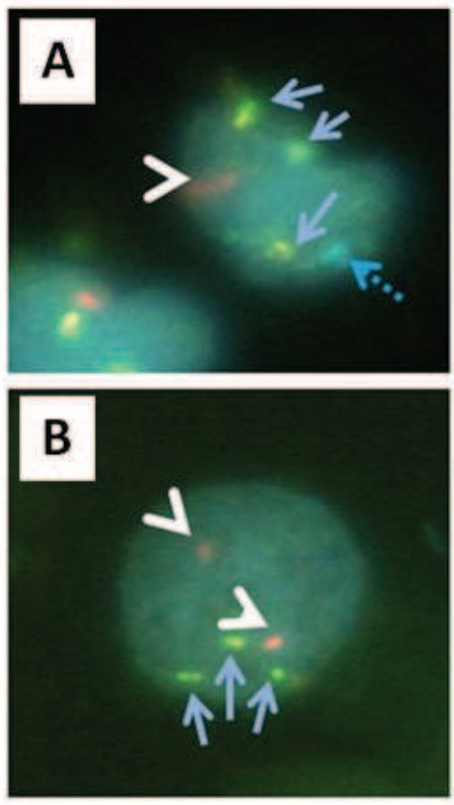

Fig. 1. Identification of trisomy 21 cells in cervical samples from trisomy 21 pregnancies. Panel A: Male trisomy 21 nucleus exhibiting X signal (white arrowhead), Y signal (broken blue arrow) and chromosome 21 signals (arrows). Panel B: Female trisomy 21 nucleus exhibiting two X signals (white arrow heads) and three chromosome 21 signals (arrows).

7. Conclusion

The development of a non-invasive genetic test for Down syndrome that would provide true genetic information without carrying risk for the progress of the pregnancy will continue to be an actively researched area in prenatal diagnosis. The trials performed so far highlight the medical and commercial potential of NIPD but the proposed techniques are not yet applicable in clinical practice. A major obstacle in the widespread application of NIPD in clinical diagnostics is that fetal DNA constitutes a small percentage of total DNA in maternal blood and intact fetal cells are even rarer. In the previous years the researchers were trying to discover Y chromosomes sequences or paternally inherited polymorphisms as targeted fetal DNA markers in maternal plasma but still there is no such a single marker that can be applied in all fetal-maternal pairs. One promising alternative approach appears to be the development of gender- and polymorphism- independent fetal DNA markers with a unique methylation pattern that will characterize the placental-derived free DNA in the maternal circulation. In parallel, the refinement of novel sequencing methods will create a

universal test for fetal aneuploidy by using maternal plasma DNA that will not depend on the presence of specific genetic polymorphisms at specific loci but on the enrichment and quantification of cffDNA in maternal peripheral blood. Also, an important goal of the ongoing research is to develop laboratory protocols with the aid of bioinformatics algorithms that will allow their application in large sample numbers. Nevertheless, large-scale studies will need to be performed to confirm the diagnostic efficacy of these methods and subsequently lead to introduction of the experimentally validated strategies into the clinical practice of fetal medicine.

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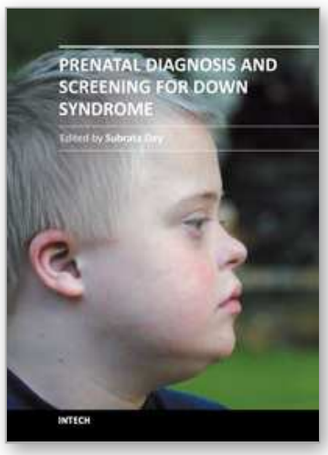

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This book provides a concise yet comprehensive source of current information on Down syndrome. Research workers, scientists, medical graduates and paediatricians will find it an excellent source for reference and review. This book focuses on exciting areas of research on prenatal diagnosis - Down syndrome screening after assisted reproduction techniques, noninvasive techniques, genetic counselling and ethical issues. Whilst aimed primarily at research worker on Down syndrome, we hope that the appeal of this book will extend beyond the narrow confines of academic interest and be of interest to a wider audience, especially parents and relatives of Down syndrome patients.

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University Campus STeP Ri
Slavka Krautzeka 83/A
51000 Rijeka, Croatia
Phone: +385 (51) 770 447
Fax: +385 (51) 686 166
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InTech China
Unit 405, Office Block, Hotel Equatorial Shanghai
No.65, Yan An Road (West), Shanghai, 200040, China
中国上海市延安西路65号上海国际贵都大饭店办公楼405单元
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